A Frameshift Variant in the Acylglycerol Kinase Gene Displays a Unique Role in the Etiology of Papillary Thyroid Carcinoma

Research Thesis

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By

Isabella V. Hendrickson

The Ohio State University

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Project Advisors: Dr. Albert de la Chapelle & Dr. Daniel Comiskey, The Department of Cancer Biology and Genetics

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ABSTRACT

In a recent study, whole-genome sequencing (WGS) of 17 families with incidence of non-medullary thyroid carcinoma led to the identification of 41 significant germline variants in 40 genes including 24 missense, five frameshift, five splice change, and seven nonsense variants. One variant, located within the Acylglycerol Kinase (*AGK*) gene, is both a frameshift and splice region variant (*AGK* c.521_522del). *AGK* encodes a mitochondrial membrane protein involved in lipid and glycerolipid metabolism by functioning as a lipid kinase to catalyze the phosphorylation of both monoacylglycerol and diacylglycerol to form lysophosphatidic acid (LPA) and phosphatidic acid (PA) respectively. Aside from functioning as a lipid kinase, AGK is a component of the TIM22 complex, which mediates the import and insertion of proteins into the mitochondrial inner membrane. Our study provides further characterization of the recently identified and validated frameshift variant, *AGK* c.521_522del, in the development of PTC. Our genetic and molecular data suggest altered expression of the encoded truncated protein results in loss-of-function, rather than a dominant negative function. However, as this is only one of many genes implicated in thyroid cancer, and its role has yet to be definitively characterized, future studies to refine the functionality of this gene as well as the additional risk of other loci will confer a better understanding of genes predisposing to thyroid carcinoma.

INTRODUCTION

Thyroid Carcinoma, a group of cancers that begin in the cells of the thyroid gland, is considered the most common malignancy of the endocrine system.[1] The thyroid is a bilobed gland that sits in the neck just beneath the windpipe. The thyroid gland produces hormones that function in the maintenance of homeostasis and thus are crucial in controlling many regulatory processes including heartrate, blood pressure, temperature, and metabolism. The thyroid gland also contributes to the maintenance of blood calcium levels, thus contributing to the functioning of nerves and muscles.[2] According to the National Cancer Institute, the number of new cases of thyroid cancer in 2019 was approximately 52,070, accounting for 3% of all new cancer cases.[3] Furthermore, in using statistical models for analysis, it was found the rate of new thyroid cancer cases has been rising about 1.9% each year over the last decade while death rates have also been rising around 0.7% each year.[4] With respect to the United States specifically, the rate of new cases has been rising since the 1990s, making thyroid cancer among the fastest growing cancers today.[5] According to the American Cancer Society, thyroid cancer as well as most other thyroid disease, such as Hashimoto's thyroiditis, are seen occurring three times more often in women than men, though the underlying factors predisposing to such are unknown at this time.[6] Furthermore, while both development and onset of thyroid cancer can occur at any age, in the U.S. such risk has been observed to peak earlier for women than men.[7] According to the American Cancer Society, risk factors for the development of thyroid cancer include gender, age, exposure to radiation, iodine levels in the diet, and family history.

As aforementioned, thyroid cancer is actually a group of cancers that begin in the cells of the thyroid. There are four main types of thyroid cancer: papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), medullary thyroid carcinoma (MTC), and anaplastic thyroid

carcinoma (ATC).[8] Differentiating between these four types depends primarily upon how the cancer cells appear under the microscope.[9] Medullary thyroid carcinoma (MTC) occurs in the C cells of the thyroid gland, which function in producing calcitonin, a hormone that when released reduces blood calcium levels.[10] Non-medullary thyroid cancer, NMTC, (PTC, FTC, ATC), derived from the follicular cells of the thyroid gland, accounts for more than 90% of all thyroid cancers.[11] Both MTC and NMTC can occur in sporadic and familial forms. Hereditary MTC accounts for 20-25% of all MTC and can either present associated with multiple neoplasia (MEN) IIA or IIB, or as pure familial MTC (FMTC).[12] The genetic predisposition for FMTC is well understood and genotype-phenotype correlations are well-defined.[13] However, the hereditary nature of and genetic predisposition to NMTC is not well established as it has only begun to emerge over the last 10 to 20 years.[14]

Although NMTC is primarily sporadic, familial aggregation does occur.[15] Based on both clinical and pathological findings, hereditary NMTC can be classified either as syndromic-associated or pure familial NMTC (FNMTC).[16] Regarding syndrome-related incidence, hereditary NMTC has been found to be associated with Cowden's Syndrome (CS), Familial Adenomatous Polyposis (FAP), Carney's Complex, and Werner's Syndrome.[17] Pure familial non-medullary thyroid cancer (FNMTC), defined as two or more affected family members, typically follows an autosomal dominant pattern of inheritance and represents 5-15% of NMTC and 3-9% of all thyroid cancers.[18] Furthermore, FNMTC is thought to cause more aggressive disease.[19] PTC, the most common histological subtype of FNMTC, often presents by itself within families and thus is termed familial PTC (FPTC).[20] Other forms of FNMTC include PTC follicular variant, FTC, and rarely ATC.[21]

Although the specific underlying genetic influence on FNMTC is not well established, case-control studies have shown that the development of both PTC and FTC is largely subject to hereditary factors (predisposing genes).[22] In studies from Utah and Sweden, first degree relatives of probands, had an 8- and 12-fold risk respectively, to develop PTC/FTC, a degree of heritability among the highest of all cancers.[23] One emerging factor known to influence genetic predisposition to PTC is the penetrance of a disease-causing variant. Of the variant genes known to strongly predispose to PTC, none account for more than a few cases.[24] Such genes are termed highly penetrant. In contrast, variant genes displaying weaker or incomplete predisposition, termed low-penetrant genes, may be more common.[25] The high heritability of PTC is likely due to a collective contribution of genes that display both high and low penetrance.[26]

Highly-penetrant genes are classically identified with methods of linkage analysis in affected families. This method aims to detect the chromosomal location of disease-causing variants based on the concept that genes residing close to one another on a chromosome remain linked during meiosis (unlikely to be separated onto distinct chromatids during recombination) and thus are more likely to be inherited together. A few loci found using this method of linkage analysis have been implicated in the genetic predisposition to thyroid cancer, including a linkage locus within chromosome 8q24 containing a maximum nonparametric linkage (NPL) score of 7.03.[27] In this locus, a small 12 Kb haplotype, located within two known coding genes, thyroglobulin (*TG*) and Src-like adaptor (*SLA*), was found segregating with PTC.[28] Within this region, a long noncoding RNA gene, *AK023948*, was found to be a candidate susceptibility gene for PTC. However, in the absence of obvious deleterious variants and because the molecular function of *AK023948* itself has not been well-defined, no credibility regarding predisposition to PTC was assumed.[29]

In yet another study of a large family displaying NMTC (11 cases of PTC and 2 cases of ATC), linkage analysis revealed a locus in chromosome 4q32 yielding a maximum NPL score of 18.5.[30] Targeted deep sequencing of this region revealed a novel single nucleotide variant (SNV) in chromosome 4q32 (4q32 A>C) that was present in all affected family members and segregated with the disease phenotype in all but one affected individual.[31] It was discovered that this highly-penetrant 4q32 A>C variant was located in an enhancer element and both impaired this enhancer region's ability to bind transcription factors and led to a downregulation in enhancer RNA (eRNA), further suggesting its implication in the predisposition to NMTC.[32] In an additional study into the 4q32 A>C variant, the Kaplan-Meier method of segregation was used to determine that individuals with such variant have a 68.9% risk to develop thyroid cancer by age 70.[33] Interestingly, this variant was not found in 38 additional NMTC families, nor was it found in 2500 sporadic PTC cases and 2500 controls, suggesting this highly-penetrant variant is ultra-rare.[34]

In another study, linkage analysis in a series of 38 PTC families followed by association studies revealed a candidate susceptibility gene in PTC, the Slit-Robo Rho GTPase activating protein 1 (*SRGAP1*) gene, within a locus on chromosome 12q14, displaying a PPL score of 30% that coincided with an NPL score of 2.8.[35] Sequencing of all exons and exon-intron boundaries of *SRGAP1* in one affected individual from each family compatible with linkage to 12q14 led to the discovery of five missense variants. Two such variants, each present within a single family, were identified as benign.[36] The three potential disease-causing missense variants were found to co-segregate with the PTC phenotype in their relevant families.[37] Further analysis into these three missense variants' function, or lack thereof, were highly suggestive of having deleterious effects. However, population data indicated low penetrance and thus the role of *SRGAP1* variants in the overall predisposition to thyroid cancer are likely minute.[38]

Because all aforementioned highly-penetrant variants displaying a strong predisposition to PTC are quite rare, linkage analysis methods may not be as useful.[39] Today, Next Generation Sequencing (NGS), which includes whole genome and whole exome sequencing methods, is used preferentially to detect high-penetrant variants predisposing to PTC.[40] In a study of a PTC family with six affected individuals, whole genome sequencing (WGS) followed by whole exome sequencing (WES) and haplotype analysis revealed a heterozygous germline missense variant in the serine/arginine repetitive matrix 2 (*SRRM2*) gene (S346P) that co-segregated with PTC.[41] Functional analysis permitted the speculation that the mutant protein affects splicing of at least one of the genes specifically expressed in the thyroid, further implicating this rare, but highly-penetrant variant in PTC predisposition.[42]

In a recent study, WGS of 17 families with incidence of NMTC led to the identification of 41 significant germline variants in 40 genes including 24 missense, five frameshift, five splice change, and seven nonsense variants.[43] Sanger sequencing was used to validate all 41 highly-penetrant variants and prove their co-segregation with NMTC.[44] Of such identified variants, one, located within the Acylglycerol Kinase (*AGK*) gene (*AGK* c.521_522del), is both a frameshift and splice region variant. In this study, the role of *AGK* in the etiology of PTC has been characterized further.

This frameshift and splice region variant in *AGK* was discovered in one large NMTC family. Within this family are four individuals affected with NMTC in at least three generations, including three cases of PTC and one case of ATC. *AGK* is located on the long arm of chromosome 7 at position 34 (7q34). The identified variant occurs in exon 9, and results in a frameshift mutation in the encoded protein's histidine residue AGK p.(His174fs). According to the US National Library of Medicine, *AGK* encodes a mitochondrial membrane protein involved in lipid and

glycerolipid metabolism by functioning as a lipid kinase to catalyze the phosphorylation of both monoacylglycerol and diacylglycerol to form lysophosphatidic acid (LPA) and phosphatidic acid (PA) respectively.[45] This encoded protein consists of 422 amino acids and has a molecular mass of about 47 kD.[46] Aside from functioning as a lipid kinase, AGK is a component of the TIM22 complex, which mediates the import and insertion of proteins into the mitochondrial inner membrane.

It has been observed that overexpression of AGK leads to increased proliferation in breast and prostate cancer through the suppression of FOXO1 (X. Wang et al., 2014) or activation of MAPK pathway (Berktas et al., 2015), respectively.[47,48] Furthermore, *AGK* somatic mutations have been discovered in several cancers, most prominently uterine cancer.[49] Defects in this gene have also been associated with mitochondrial DNA depletion syndrome 10.[50] According to the GTEx Database, in looking at tissue specific expression of *AGK,* there is ubiquitous expression in the thyroid (RPKM 4.79, TPM 20.88) and 26 other tissues.[51] The current hypothesis is that the frameshift and splice region variant in *AGK* results in gene expression changes and/or a dominant negative function that alters the metabolism of thyroid cells in the development of FNMTC.

METHODS

Cell Lines: Cells of a PTC-derived cell line, TPC1, were incubated in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) (Thermo Fisher), 1 X antibiotic-antimycotic (Thermo Fisher), and 1 X Plasmocin prophylactic (InvivoGen) at 37˚C in humidified air with 5% CO_2. This cell line was obtained from Rebecca Schweppe (University of Colorado Cancer Center, Denver, CO).

PCR: PCR of wild type and variant *AGK* was performed using AmpliTaq Gold (Thermo Fisher) under standard PCR conditions.

Generation of Plasmid Constructs: The coding sequence of wild-type *AGK* (position 40-1308) was PCR amplified using AccuPrime Taq (Thermo Fisher). The product was then purified and cloned into XhoI and HindIII sites of the pCMV6-Entry Tagged Cloning Vector. All cloning was performed using T4 DNA Ligase (New England BioLabs). The frameshift variant was induced using the GeneArt Site-Directed Mutagenesis PLUS System (Thermo Fisher).

Transient Transfection: TPC1 cells incubating in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) (Thermo Fisher) were transiently transfected with 10 μg of either pCMV6-Empty, AGK wild-type, or AGK mutant plasmid DNA using Lipofectamine 2000 (Thermo Fisher) and Opti-MEM I Reduced Serum Medium. The cells were given fresh DMEM with 10% FBS after 4 hours and then harvested after 24 hours.

Western Blotting: 24 hours after transient transfection, TPC1 cells were lysed in 1 X RIPA Lysis and Extraction Buffer (Thermo Fisher) with 1 X Halt protease/phosphatase inhibitor (Thermo Fisher). Equal amounts of protein were boiled and eluted in 1 X Laemmli buffer (Bio-Rad) containing 2-mercaptoethanol (Sigma-Aldrich). Proteins were then loaded onto a 4-20% TGX Stain-Free precast SDS-PAGE gel with All Blue Protein Standards (Bio-Rad). Gels ran at 100 V for 10 minutes, then 150 V for 60 minutes. Gels were activated under UV light using a ChemiDoc (Bio-Rad). Total protein was visualized under UV light using a ChemiDoc (Bio-Rad). Following total protein visualization, gels were transferred to low-fluorescence polyvinylidene fluoride membranes using an RTA transfer kit (Bio-Rad) and Trans-Blot Turbo system (Bio-Rad). Total protein was visualized on the blot under UV light using a ChemiDoc (Bio-Rad). Blots were then blocked in 5% non-fat milk in tris-buffered saline with Tween 20 (TBS-T) at room temperature for 1 hour. Following blocking, blots were probed with unconjugated monoclonal primary antibody in 5% non-fat milk (FLAG [M2] Sigma, GAPDH-HRP [0411] Santa Cruz) at 4˚C overnight. Blots were then washed in TBS-T and incubated in conjugated polyclonal secondary antibody at room temperature for 1 hour (anti-mIgG-HRP Sigma or anti-rIgG-HRP Cell Signaling). Blots were washed in TBS-T and Clarity Enhanced Chemiluminescence (ECL) (Bio-Rad) was performed. Chemiluminescence was visualized using a ChemiDoc (Bio-Rad) and through exposure of Blue Basic Autorad Double Emulsion Film (GeneMate). To re-probe, blots were first washed in TBS-T, then quenched in 15% H_2O_2 in phosphate-buffered saline (PBS) (Thermo Fisher) at room temperature for 15 minutes.

Microscopy: Coverslips were exposed to UV light for 15 minutes on each side then treated with poly-D-lysine (0.1 mg/ml) for 5 minutes at room temperature. Coverslips were then washed with

PBS and dried for 2 hours. For observation of endogenous AGK expression, 250,000 TPC1 cells were seeded on these pretreated coverslips. After 24 hours, cells were washed in PBS, fixed in 4% paraformaldehyde for 5 minutes, and permeabilized in 0.25% Triton X-100 for 15 minutes. After washing with PBS, the cells were blocked in 10% donkey serum in a humidified chamber at 4˚C overnight. Cells were then incubated with unconjugated primary antibodies (AGK (HPA020959 Atlas, alpha tubulin (DM1A) Abcam) in 5% donkey serum in a humidified chamber at 4˚C overnight. After washing with PBS supplemented with 0.1% of 10% Tween (PBS-T), cells were incubated in conjugated secondary antibody (anti-mIgG Alexa Fluor 647 Thermo Fisher or anti-rIgG Alexa Fluor 488 Thermo Fisher) in 5% donkey serum at room temperature for 1 hour in the dark. After washing with PBS-T, coverslips were mounted on microscope slides with 1 drop of Diamond ProLong Antifade with 4',6-diamidino-2-phenylindole (Thermo Fisher) and cured overnight in the dark at room temperature. For observation of exogenous wild-type and truncated AGK expression, 250,000 TPC1 cells were seeded on pretreated coverslips. After 24 hours, 250,000 TPC1 cells, transiently transfected over 24 hours with either pCMV6-Empty, AGK wild-type, AGK mutant plasmid DNA, were seeded on these pretreated coverslips. After 24 hours, cells were either stained with 100 mM MitoTracker Red CMXRos (Thermo Fisher) in DMSO for 15 minutes, or left unstained. All cells were then washed in PBS, fixed in 4% paraformaldehyde for 5 minutes, and permeabilized in 0.25% Triton X-100 for 15 minutes. After washing with PBS, the cells were blocked in 10% donkey serum in a humidified chamber at 4˚C overnight. Cells were then incubated with unconjugated primary antibodies (alpha tubulin [DM1A] Abcam, or FLAG [M2] Sigma) in 5% donkey serum in a humidified chamber at 4˚C overnight. After washing with PBS supplemented with 0.1% of 10% Tween (PBS-T), cells were incubated in conjugated secondary antibody (anti-mIgG Alexa Fluor 647 Thermo Fisher or anti-rIgG Alexa Fluor 488

Thermo Fisher) in 5% donkey serum at room temperature for 1 hour in the dark. After washing with PBS-T, coverslips were mounted on microscope slides with 1 drop of Diamond ProLong Antifade with 4',6-diamidino-2-phenylindole (Thermo Fisher) and cured overnight in the dark at room temperature. Cells were visualized and imaged using a FV3000 confocal laser scanning microscope (Olympus).

Proliferation Assay: Equal amounts of the tetrazolium salt, WST-8 ([2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophneyl)-2H-tetrazolium, monosodium salt)]), were added to TPC1 cells seeded in a 24-well plate and transiently transfected over 24 hours with reported plasmids as described above. Proliferation was measured according to the extracellular reduction of WST-8, which results in an orange colored dye, formazan. The amount of formazan produced, measured as the absorption at 450 nm by a SpectraMax M2e Multilabel Microplate Reader (Marshall Scientific), is directly proportional to the number of viable cells.

Flow Cytometry: Following transient transfection, TPC1 cells were washed twice with PBS and then resuspended in PBS. Cell suspensions were passed through a 100 μm cell strainer into polystyrene tubes and stained with propidium iodide. Cell suspensions were then incubated on a shaker in the dark for 15 minutes and analyzed on a LSR II flow cytometer (BD Biosciences).

Statistical Analysis: All graphical data are represented by mean±SEM. All p values reported are calculated from a two-sided t-test.

RESULTS

AGK c.521_522del shows co-segregation with PTC in one large NMTC family.

In a previous study, whole genome sequencing (WGS) of 17 families with incidence of non-medullary thyroid cancer (NMTC) led to the identification of 41 significant germline variants.[52] Sanger sequencing validated all 41 variants, and proved their co-segregation with NMTC. 5 of these 41 variants were found to co-segregate with PTC in one of these 17 families. All but one of such identified variants are either missense or nonsense variants resulting from single nucleotide substitutions (**Table 1**). The other variant, located within exon 9 of the Acyglycerol Kinase (*AGK*) gene, is a both a frameshift and splice region variant (*AGK* c.521_522del). Like the other 4 variants, *AGK* c.521_522del displays co-segregation with all three PTC cases in this single NMTC family, as well as one ATC case (**Figure 1**). This variant was also identified in a family member that had a thyroidectomy due to prior presence of a benign thyroid disease. This variant was not found in any unaffected family member available for sequencing or linkage analysis (**Figure 1**). In addition to its co-segregation with PTC and the detrimental nature of frameshift variants, this variant posed as an interesting candidate to characterize further due to the ubiquitous expression of *AGK* in thyroid tissue (RPKM 4.79, TPM 20.88).[53]

Sanger sequencing and Western blotting analysis validate a truncated protein.

In the previous study, Sanger sequencing was performed to validate the incidence of all identified 41 variants, as well as their co-segregation with PTC in each respective NMTC family. Sanger sequencing of *AGK* variant carriers confirmed the presence of a frameshift variant (**Figure 2A**). This frameshift variant, *AGK* c.521_522del, located in exon 9 in *AGK,* and within a histidine residue of the encoded protein AGK p.(His174fs), results in the protein truncating after a tyrosine

residue (**Figure 2B**). We generated this truncated protein in an expression plasmid and transiently transfected a PTC-derived cell line, TPC1, with the truncated plasmid DNA. Western blotting was performed with proteins harvested from the transiently transfected cells in order to confirm the identity of the truncated protein as compared to the wild-type (**Figure 2C**). The wild-type AGK protein plus the FLAG tag consisted of 432 amino acids was expected to have a molecular weight of around 48.34 KDa.[54] The truncated AGK protein, AGK p.(His174fs), plus the FLAG tag consisted of 184 amino acids and was expected to have a molecular weight of around 20.31 KDa. These predicted values were observed in the Western blot. The tagged wild-type and truncated AGK proteins were observed on the blot around 50 KDa and between 20 and 25 KDa, respectively (**Figure 2C**). Also observed through Western blotting analysis was a discrepancy between the expression levels of the wild-type and truncated AGK proteins. The wild-type protein appears to have a much higher level of expression than the truncated protein (**Figure 2C**).

Overexpression of *AGK* and proliferation.

In order to determine if overexpression of the variant form of *AGK* (*AGK* c.521_522del) led to increased proliferation, we performed colorimetric assays using TPC1 cells transiently transfected with either *AGK* wild-type, *AGK* c.521_522del, or empty pCMV6 plasmid DNA over 24 hours. Proliferation was measured through the extracellular reduction of WST-8, which was added in equal amounts to the cells. The reduction of WST-8 produces an orange colored dye, formazan. 450 nm absorption of formazan indicated the number of viable cells. We found that overexpression of the truncated form of AGK resulted in decreased proliferation of TPC1 cells relative to the empty pCMV6 vector (**Figure 3A**). Through conducting cell cycle analysis using flow cytometry, we determined overexpression of the truncated form of AGK did not result in a significantly higher

percentage of TPC1 cells existing in both S and G2/M phases relative to either the empty pCMV6 vector or wild-type form (**Figure 3B**). These results suggest the AGK truncated protein does not gain a dominant negative function leading to an alteration in metabolic activity of thyroid cells in the development of FNMTC.

Localization of both wild-type and variant AGK.

According to the Human Protein Atlas, in studies using HEK 293, U-2 OS, and U-251 MG cell lines, AGK mainly localized to the mitochondria.[55] Before attempting to visualize how the truncated protein localizes in the cell with respect to the mitochondria, we conducted immunofluorescence staining to visualize AGK's endogenous expression in the thyroid cancer-derived cell line, TPC1. We discovered the protein has a more pan distribution than what was expected, given the previous studies conducted with other cell lines (**Figure 4**). In looking at the DAPI and alpha-tubulin staining, AGK was not restricted to either nuclear or cytoplasmic cellular compartment, again revealing a more pan distribution (**Figure 4**). In order to characterize if the potential role of loss-of-function in the etiology of PTC manifests itself in altered expression within or surrounding the mitochondria, we performed immunofluorescence staining on TPC1 cells transiently transfected with either *AGK* wild-type, *AGK* c.521_522del, or empty pCMV6 plasmid DNA over 24 hours. Due to non-specific binding of the anti-FLAG antibody, evident in our control samples, our results must be considered inconclusive (data not shown).

DISCUSSION AND FUTURE DIRECTIONS

In a previous study, Sanger sequencing was performed to validate that a frameshift variant in the acylglycerol kinase (*AGK*) gene, identified by WGS, co-segregating with PTC in a single FNMTC family.[56] This frameshift variant, *AGK* c.521_522del, occurs in a histidine residue [AGK p.(His174fs)] resulting in the protein truncating after a tyrosine residue. In the present study, we have attempted to further characterize the role of *AGK* c.521_522del in the etiology of PTC. As mentioned previously, *AGK* encodes a lipid kinase located within the inner mitochondrial membrane that functions in both lipid and glycerolipid metabolism. The *AGK* frameshift variant, *AGK* c.521_522del, is located within the region that encodes this protein's diacylglycerol kinase catalytic (DAGKc) domain. This conserved DAGKc domain mediates AGK's kinase activity in lipid metabolism through binding ATP and phosphorylating target molecules, suggesting any consequential changes in gene expression could alter the metabolism of thyroid cells in the development of FPTC.[57]

Through Western blotting analysis, we showed there was differential expression of the wild-type and truncated AGK proteins. The wild-type AGK protein displayed a higher level of expression than the truncated AGK protein, suggesting a potential discrepancy in relative stability between the two. Since the mutant protein truncates after a tyrosine residue, and thus only contains 175 of the 422 amino acids present in the wild-type AGK protein, it is possible that a loss in secondary and/or tertiary structure is responsible for this instability. This putative instability of the truncated AGK protein could also be due in part to an inability to associate with other molecules, such as those that mediate its association with the inner mitochondrial membrane, which could be assessed through co-immunoprecipitation. In order to definitively characterize the differential gene expression profiles between *AGK* c.521_522del and the wild-type, quantitative PCR should be

performed. Regardless, these findings allowed us to hypothesize that this frameshift in *AGK* resulting in gene expression changes could lead to a loss-of-function or a dominant negative function that alters the metabolism of thyroid cells in the development of familial PTC.

In other studies, it has been found that overexpression of AGK leads to increased proliferation and tumorigenicity in breast and prostate cancer through the suppression of the FOXO1 transcription factor (X. Wang et al., 2014), or cross talk with EGFR and thus activation of MAPK pathway (Berktas et al., 2015), respectively.[58,59] In order to identify whether or not the truncated AGK protein gains a dominant negative function leading to tumorigenicity in the development of PTC, we performed proliferation assays. Surprisingly, we found that overexpression of the truncated AGK protein resulted in decreased proliferation of cells from a thyroid cancer-derived cell line (TPC1). Furthermore, through performing cell cycle analysis, we determined that overexpression of the truncated AGK protein did not result in a significantly higher percentage of TPC1 cells existing in both S and G2/M phases relative to either the empty pCMV6 vector or wild-type AGK protein. These results seem to suggest that, rather than gaining a dominant negative function leading to tumorigenicity in PTC, *AGK* c.521_522del has a protective function against the development of PTC. It is, however, possible that this overexpression system may not recapitulate what is happening on an endogenous level. For these studies, either patient lymphoblast cells or isogenic CRISPR-Cas9-engineered cell lines would need used to determine the phenotype of this variant. Furthermore, this frameshift variant in *AGK* resulting in altered gene expression could still lead to a loss-of-function relevant in the development of PTC.

A potential loss-of-function in the AGK mutant protein could alter the integrity of the mitochondria. However, the truncated AGK protein likely still associates with the inner

mitochondrial membrane because it retains its N-terminal mitochondrial localization sequences. Defects in *AGK* resulting in loss-of-function have been identified as the causative agent responsible for the development of Mitochondrial DNA Depletion Syndrome 10 (MTDPS10). MTDPS10, commonly known as Senger's Syndrome, is an autosomal recessive mitochondrial disorder characterized by the presence of many conditions including cataracts, cardiomyopathy, and skeletal myopathy.[60] *AGK*'s role in the development of MTDPS10 has been characterized as two-fold in accordance with the encoded protein's two completely independent functions. First, AGK's functionality as a lipid kinase, and thus its catalytic activity, was found to play a central role in maintaining the structural integrity of the mitochondrial cristae.[61] Because mitochondria are known to trigger apoptotic cell death through cristae remodeling and fragmentation, AGK's enzymatic functionality becomes vital in a cell's apoptotic resistance. Mutations in *AGK* leading to loss-of-function of the encoded protein have been noted as the causative factor responsible for abnormal cristae morphology identified in MTDPS10 patients' cells.[62] Independent of its catalytic activity, AGK's role as a component of the TIM22 complex has also been identified in the etiology of MTDPS10. Functional AGK associated with TIM22 and TIM29 to form the TIM22 complex, which mediates both the transport and integration of multi-pass transmembrane proteins, such as the adenosine nucleotide translocator 1 (ANT1) protein, into the inner membrane of the mitochondria.[63] Loss of AGK was found to impede the assembly of the TIM22 complex, which explains the decreased expression of ANT1 observed in MTDPS10 cells.[64] It is possible that one or both of such consequences involved in the development of MTDPS10 also arise in the tumorigenicity of PTC due to a loss-of-function of the truncated AGK protein.

We performed immunofluorescence staining on the PTC-derived cell line, TPC1, to identify if perhaps a loss-of-function of the truncated AGK protein manifests in altered expression

within or surrounding the mitochondria. Given evidence of non-specific binding in our control samples, our results were inconclusive. In order to further characterize the potential consequences of a loss-of-function of the truncated AGK protein, studies into the complete loss-of-function, apoptosis, and TIM22 complex functionality should be performed. Knockdown of AGK expression in TPC1 cells using either siRNA or shRNA could be conducted to study the potential impact of loss-of-function. Global expression profiling could be conducted with patients' cells to identify differential expression patterns between the wild-type and truncated AGK proteins upon being subjected to apoptotic stimuli. Western blotting could also be performed to identify differences between the wild-type and truncated AGK proteins in their expression of certain apoptotic markers, such as cytochrome c. In terms of TIM22 complex functionality, co-immunoprecipitation analysis should be performed to identify if the truncated AGK protein is able to interact with both TIM22 and TIM29 in order to form the translocator complex. Furthermore, since ANT1 relies upon TIM22 for its insertion into the inner mitochondrial membrane, and because it is directly involved in metabolism through functioning as an ATP/ADP exchanger, its role in the development of PTC should also be considered.

Overall, our study provides further characterization of the recently identified and validated frameshift variant, *AGK* c.521_522del, in the development of PTC. Our genetic and molecular data suggest altered expression of the encoded truncated protein results in loss-of-function, rather than a dominant negative function. However, as this is only one of many genes implicated in thyroid cancer, and its role has yet to be definitively characterized, future studies to refine the functionality of this gene as well as the additional risk of other loci will confer a better understanding of genes predisposing to thyroid carcinoma.

FIGURES AND TABLES

Chromosome	Position	Reference	Alternate	Gene	Class	Affected	Unaffected
3	50684626	G	T	*MAPKAPK3*	Stop – Gained	3 of 3	0/1
4	74270076	T	A	*ALB*	Missense	3 of 3	0/1
7	141321533	CAT	C	*AGK*	Frameshift & Splice Region	3 of 3	0/1
14	55836494	G	A	*ATG14*	Missense	3 of 3	0/1
19	57932175	G	T	*ZNF17*	Stop – Gained	3 of 3	0/1

Table 1: *AGK* c.521_522del is One of the 5 Variants Identified in One NMTC Family.
In a previous study of 17 families with incidence of non-medullary thyroid cancer (NMTC), whole genome sequencing (WGS) identified 41 germline variants in 40 candidate genes predisposing to thyroid cancer. Shown here are 5 of such identified variants, which were discovered within a single family. The reference nucleotide(s) denotes the wild-type genotype at the given position, whereas the alternate represents the variant. Within the family, all individuals with PTC (affected) are carriers of all five variants. Family members available for analysis and unaffected by PTC do not carry any of the identified variants. Therefore, these 5 variants display co-segregation with PTC in this single NMTC family.[65]

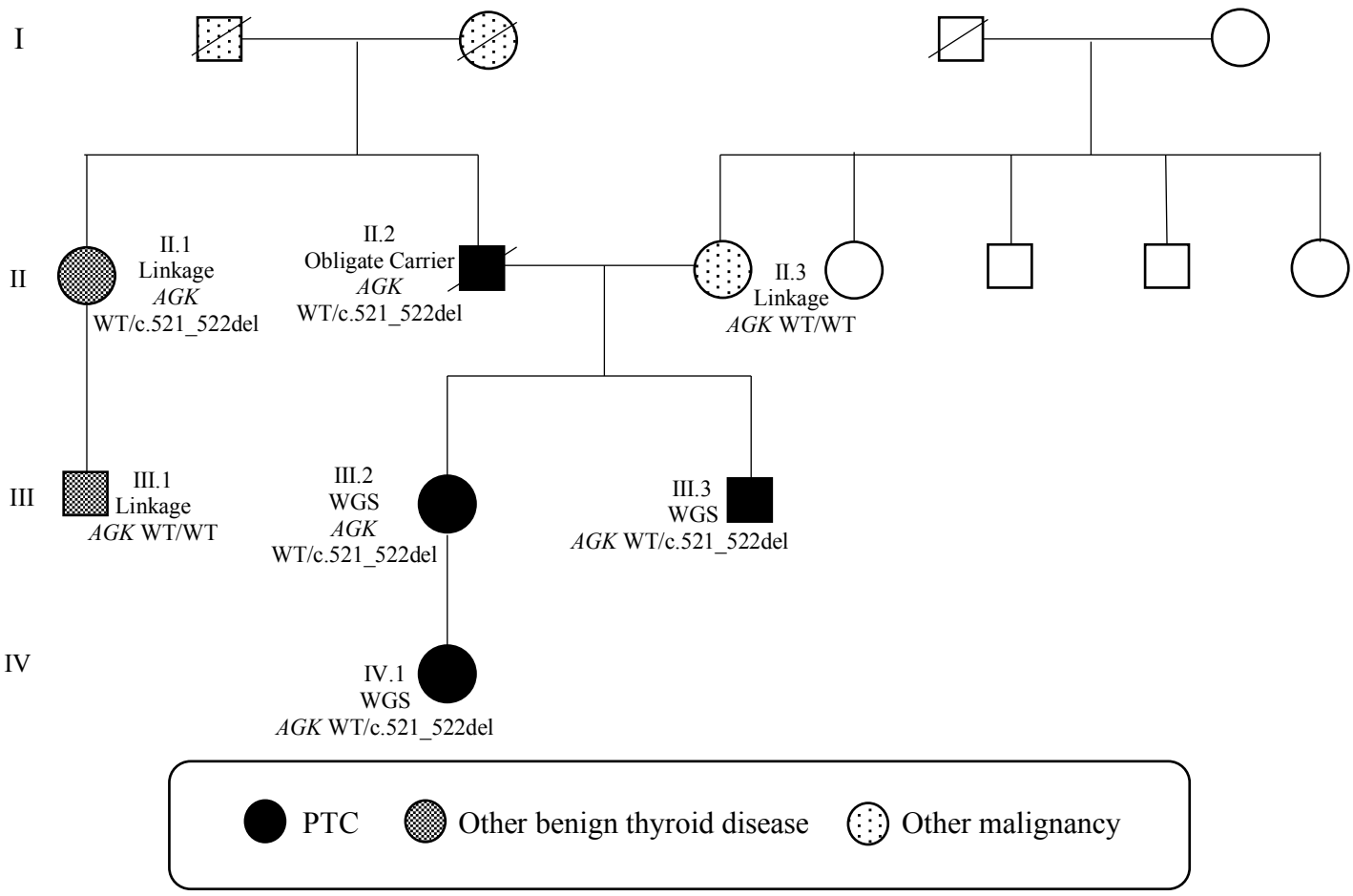

Figure 1: Pedigree of the NMTC family in Which *AGK* c.521_522del Displays Co-Segregation with PTC.

Pedigree of the non-medullary thyroid cancer (NMTC) family in which *AGK* c.521_522del cosegregates with all three cases of PTC. Family structure is indicated by the pedigree map. Squares represent males, and circles represent females. Generations are labeled using Roman numerals. Individuals are represented by both their generation and order in which they appear. Deceased family members are indicated with a diagonal line. Individual II.2 was affected with ATC.[66]

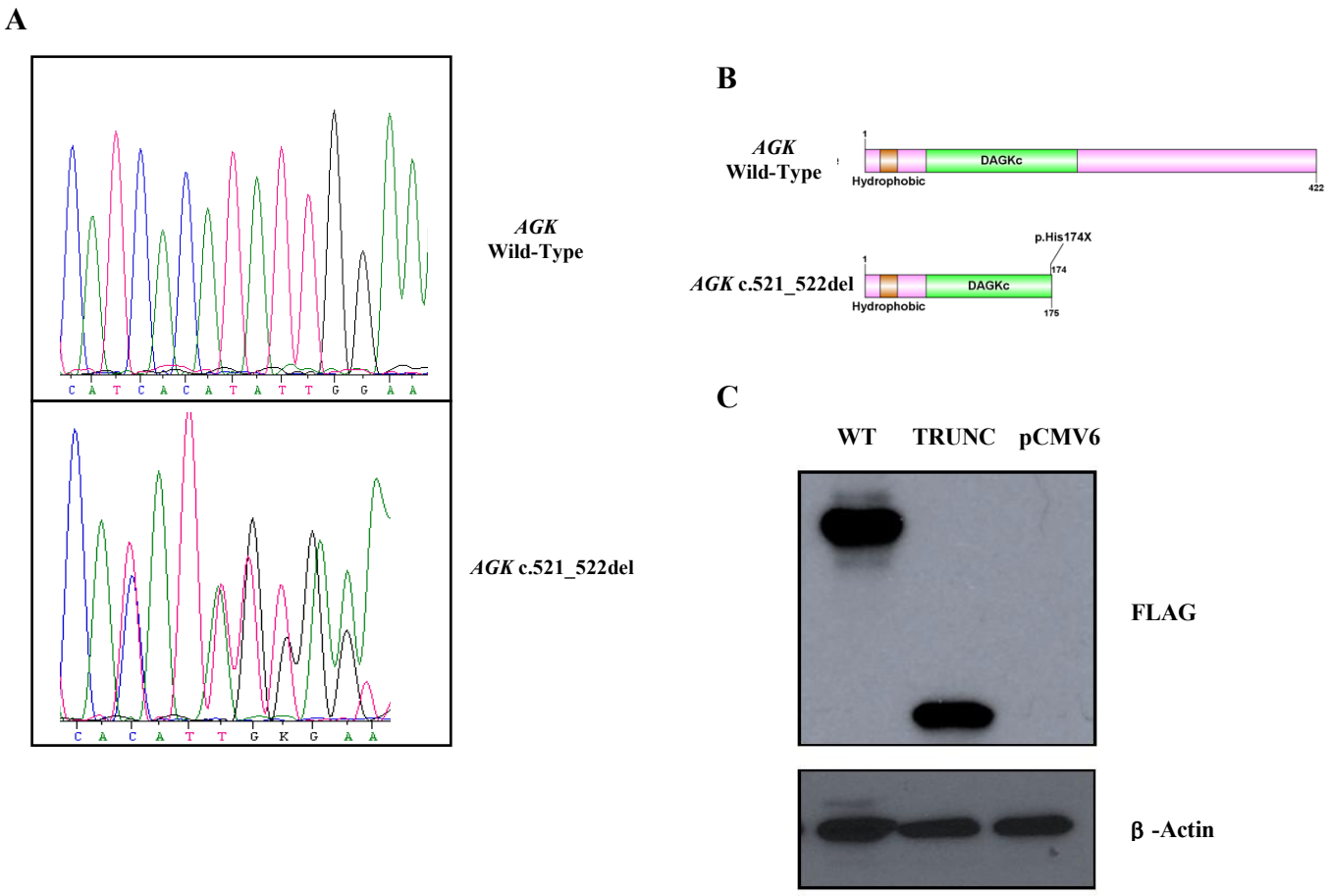

Figure 2: The Frameshift Mutation, *AGK* c.521_522del, Occurs in a Histidine Residue, [AGK p.(His174fs).] Resulting in the Encoded Protein Truncating After a Tyrosine Residue.
(**A**) Sanger sequencing data used in a previous study to identify *AGK* as one of 41 rare high penetrant variants predisposing to PTC. Sequencing data from individual II.3, unaffected by PTC nor *AGK* c.521_522del, is compared to sequencing data from individual III.2, affected by PTC and *AGK* c.521_522del. (**B**) Linear schematic of the wild-type and truncated AGK protein forms. Functional groups and the frameshift variant are labeled. The hydrophobic region is labeled in orange and the diacylglycerol kinase catalytic (DAGKc) domain is labeled in green. (**C**) Representative Western blot of cells transfected with *AGK* constructs, *AGK* wild-type (WT), *AGK* c.521_522del (TRUNC), and pCMV6 empty vector is shown.

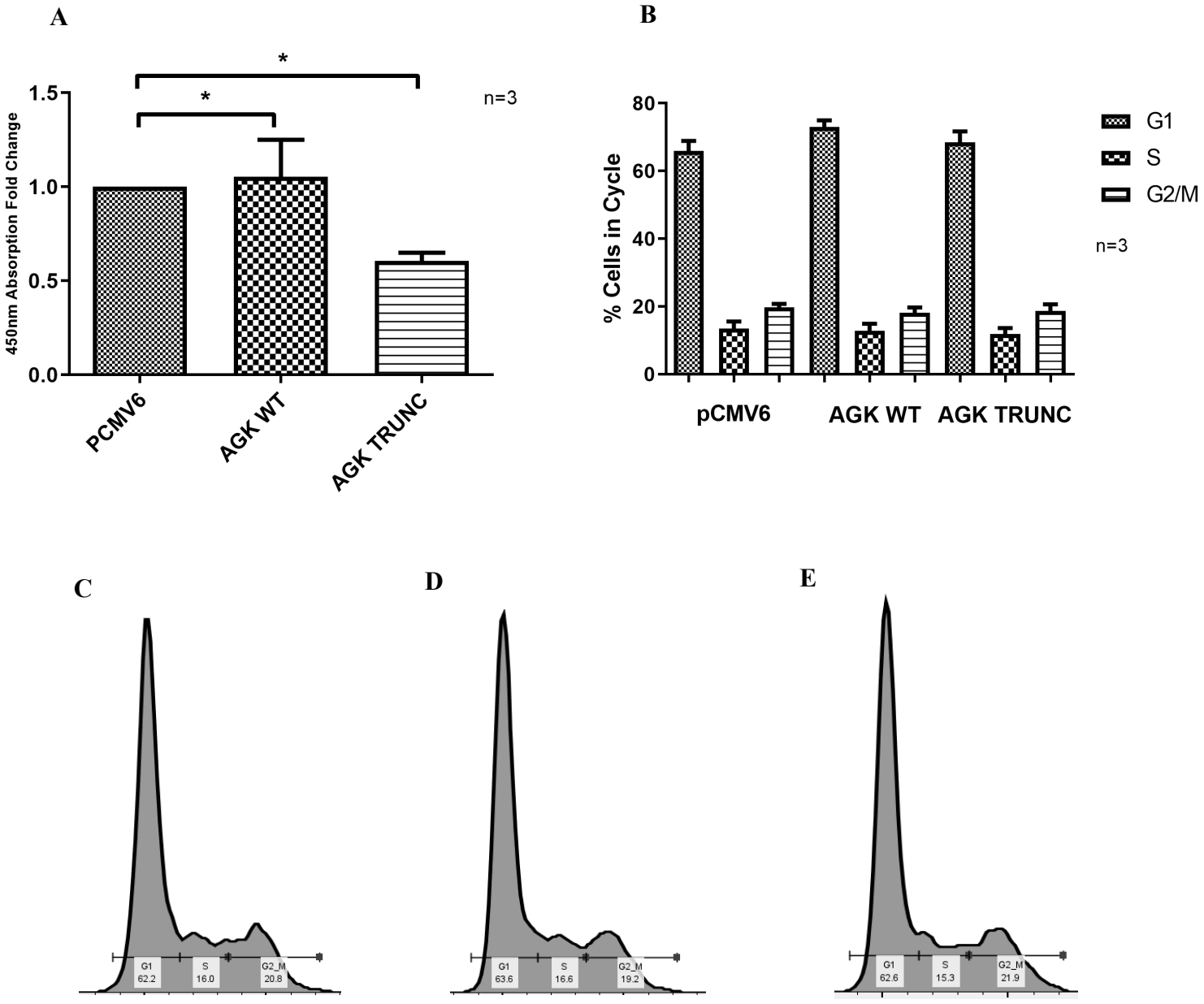

Figure 3: Proliferation Assays and Cell Cycle Analysis.
WST-8 absorption and flow cytometry identify differential proliferation of TPC1 cells transiently transfected with plasmid DNA over 24 hours. (**A**) Proliferation was measured as the absorption of formazan at 450 nm, which is directly proportional to the number of viable cells. Quantitation of the absorption was normalized according to the empty pCMV6 vector. Results are shown as mean±SEM, n=3. *$p<0.05$ (two-tailed t test). **(B)** The percentage of TPC1 cells transiently transfected with plasmid DNA at each stage of the cell cycle. Results are shown as mean±SEM, n=3. Flow cytometry histograms from TPC1 cells transiently transfected with either the (**C**) pCMV6 empty vector (pCMV6), (**D**) *AGK* wild-type (AGK WT), or (**E**) *AGK* c.521_522del (AGK TRUNC) for 24 hours. Cells were stained with propidium iodide. The percentage of cells at each stage of the cell cycle were obtained from these histograms.

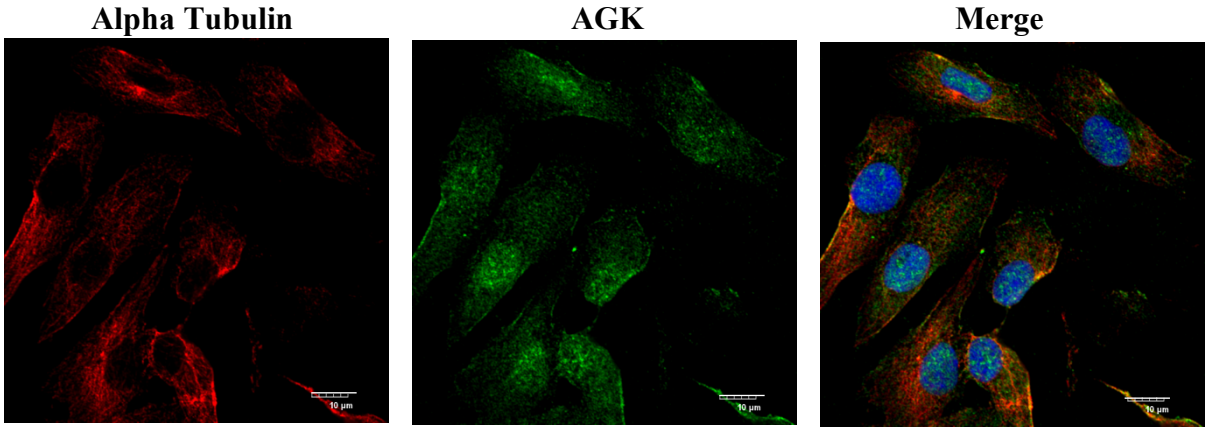

Figure 4: Confocal Image of Endogenous AGK Expression in TPC1 Cells.
Endogenous expression of the AGK protein in a PTC-derived cell line, TPC1. Immunofluorescence staining was performed in order to visualize this expression under a confocal microscope. All images were taken under 60 X magnification. The scale bar, denoted in white, represents a distance of 10 um.

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